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14. ABSTRACT: In this project, we propose to develop a new drug delivery vehicle based on dendrimer nanotechnology for personalized medicine. This new class of nanoplatfroms contains imaging probe and molecular medicine with a cancer-specific targeting capability which is able to target cancer cells, monitor drug delivery and tumor response to achieve a "see and treat" strategy as a new concept of molecular medicine. Specifically, One Partner PI's lab will make dendrimers bearing functional handles to conjugate with chelating agents provided by the Initiating PI's lab for PET imaging and therapeutic peptides provided by another Partner PI's lab for the treatment of aggressive prostate cancer. As the continuation of last year's work, we have designed and successfully prepared a theranostic small molecule drug conjugate – T-SMDC which consists of tumor-specific ligand, PET imaging moiety, and cytotoxic drug. The resulted theranostic small-molecule drug conjugate (T-SMDC) remains the PSMA binding affinity and exhibits PSMA-dependent anti-cancer toxicity. To date, we have developed the radiochemistry procedure to label the T-SMDC with <sup>68</sup> Ga efficiently. We have started evaluating its therapeutic performance along with the concurrent PET imaging in PSMA <sup>+</sup> tumor bearing mice.					
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## Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	14
Reportable Outcomes.....	14
Conclusion.....	14
References.....	14
Appendices.....	N/A

## INTRODUCTION

This project combines the recent advances in prostate cancer (PCa) research from three different labs integrated with a strong interest and dedication to develop a new molecular medicine approach towards the eventual cure of PCa. Like other cancer types, the current available therapeutic regimens for metastatic PCa are not PCa specific. With respect to PCa cells harboring various genetic alterations, the development of small molecular agents targeting these genetic defects to achieve a better therapeutic efficacy is foreseeable. In this project, we propose to develop a new drug delivery vehicle based on dendrimer nanotechnology for personalized medicine. This new class of nanoplatforms contains imaging probe and molecular medicine with a cancer-specific targeting capability which is able to target cancer cells, monitor drug delivery and tumor response to achieve a “see and treat” strategy as a new concept of molecular medicine. This platform system will be flexible to adopt any new cell targeting molecule or any therapeutic agents. Specifically, Dr. Simanek’s lab will make dendrimers bearing functional handles to conjugate with chelating agents provided by Dr. Sun’s lab for PET imaging and therapeutic peptides provided by Dr. Hsieh’s lab for the treatment of aggressive PCa.

## BODY

With the ultimate goal to generate a new class of dendrimer-based theranostic agents for aggressive PCa, we have arranged four Specific Aims as indicated in Statement of Work (SOW). Our work in the first year focused on Tasks 1 – 3 to accomplish **Aim 1** and **Aim 2**.

**Aim 1:** To construct dendrimer conjugates containing specific cell permeation peptides, peptide therapeutic(s) and a bifunctional chelator for PET imaging

*Task 1 (Months 1 – 24): Synthesis and Characterization of Dendrimers - Scaffold Library*

Please see Dr. Simanek’s annual report for the progress of this Task

*Task 2 (Months 1 – 12): Synthesis & Characterization of CB-TE2A-based Bifunctional Chelator*

Accomplished in the first two years. Further work has been carried out to extend the accomplishment of Task in the 3<sup>rd</sup> year.

**Aim 2:** To select potent compounds with screening systems based on specific mechanism(s) of action

*Task 3 (Months 1 – 24). Selection of therapeutic peptides using high throughput assays*

Please see Dr. Hsieh’s annual report for the progress of this Task

**Aim 3:** To determine the biodistribution, pharmacokinetics, and potential cytotoxicity *in vivo*

*Task 4 (Months 13 – 24): Radiochemistry and in vitro assay of the synthesized theranostic agents (Sun/Hsieh)*

An imaging functionality has been incorporated into a drug conjugate platform containing an anti-cancer drug (DM1) and prostate specific membrane antigen (PSMA) for prostate cancer theranostic application.

*Task 5 (Months 9 – 30): In vivo and PET/CT imaging evaluation of the synthesized theranostic agents (Sun)*

The therapeutic effect and imaging performance of the theranostic platform are under evaluation in prostate cancer mouse models.

**Aim 4: To evaluate the therapeutic efficacy using various pre-clinical models (Hsieh/Sun/Simanek)**

*Task 6 (Months 24 – 36): Preparation of dendrimer conjugates for Aim 4 (Simanek). See Task 1.*

*Task 7 (Months 18 – 30): Target validation of dendrimer conjugates (Hsieh)*

*Task 8 (Months 25 – 36): Therapeutic efficacy of dendrimer conjugates (Hsieh/Sun)*

Delayed – A 12-month no-cost extension (NCE) has been filed in order to accomplish the tasks from 09/01/2015 to 08/31/2016.

Justification: This NCE is requested to accomplish the unfinished tasks proposed in Aims 3 & 4 of the project. The delay of the synthetic work has been explained in Dr. Simanek's NCE request: "The request for the no-cost extension is based both on scientific and personnel issues. The scientific issues focus on delays inherent to the efforts; targets based on literature precedent proved more challenging to deliver than what have been anticipated. A personnel issue also delayed advance; the post-doc supported on the efforts was replaced with a senior graduate student whom should be able to deliver on the proposed statement of work more rapidly." In addition, Dr. Hsieh's group and my lab have experienced unexpected inconsistency of the xenograft models in the past year. We have to delay the proposed in vivo testing of the proposed experiments for further verification.

**Key Progresses Made in Partner PI's Labs in the 3<sup>rd</sup> Year**

**Dr. Hsieh's lab:** Although we obtained some promising results from the P10 therapeutic peptide conjugated with dendrimer unit (CSIV-81) from last year, Dr. Simanek decided to modify the unit by adding targeting peptide R11. He generated a variety of conjugates. However, the results appeared not so consistent. We are still working on change chemical design to generate better compound. Nevertheless, we went ahead to deliver CSIV-81 into animal using mini-pump to examine its potential efficacy. Initially, we had some problem for animal model because source of animal was infected. So, we have to delay until the colony reestablished. In the first experiment, we delivered 10 mg/kg of CSIV-81, due to the limitation from the synthesis, into animal bearing prostate cancer cells and examined its effect on AKt inactivation based on phosphorylation pattern. However, the negative result was obtained. Now, we are doing dose escalation in hope to reach effective dosage.

**Dr. Simanek's lab:** Advances during this period include the description and communication [Ji et al. in *Molec. Pharmaceutics*, 2015, 12(8): 2924-7] of the proposed hydrazone chemistry for linking groups to dendrimers through a triazinyldiazine. Here, computational and experimental studies revealed an inversion of stability of these constructs at more acidic pH when compared to acyl hydrazone equivalents. The stability of different tethering groups was assessed and was found to be tunable. That is, if the therapeutic peptide bears an aryl aldehyde such as a *p*-formylbenzoyl group on the N-terminus, release is expected to be notably retarded

than a peptide bearing an *N*-terminal levulinic acid. Aromatic ketones fall in the middle. The ability to tune release of the cargo is hypothesized to be critical for these studies.

The lead monomeric constructs presented an *p*-aminoacetophenone group on a triazine that displayed both a targeting R<sub>11</sub> sequence and the therapeutic peptide. A dendrimer presenting multiple hydrazinyltriazines has been prepared and conjugation studies are ongoing. These conjugates could include the dual display (monomeric) group described, or a mixture of therapeutic and targeting peptides (mole ratios of 100:0 --> 0:100) with suitable aldehyde/ketone groups.

Efforts to rely on non-releasable constructs--that is, dendrimers with maleimides to react with cysteine-containing peptides--proved challenging on multiple levels including physicochemical properties of the platform, stability of the maleimides, and challenges to characterization. The construct delivered proved to have no biological activity. This strategy has been abandoned.

The poly(arginine) sequence has proven to be markedly problematic in both synthesis and characterization. These observations were independently confirmed by studies completed in the Pellois laboratories of Texas A&M University, who pursued these constructs for independent purposes. Alternatives to this sequence are being considered including the prostate-specific targeting group, DUPA.

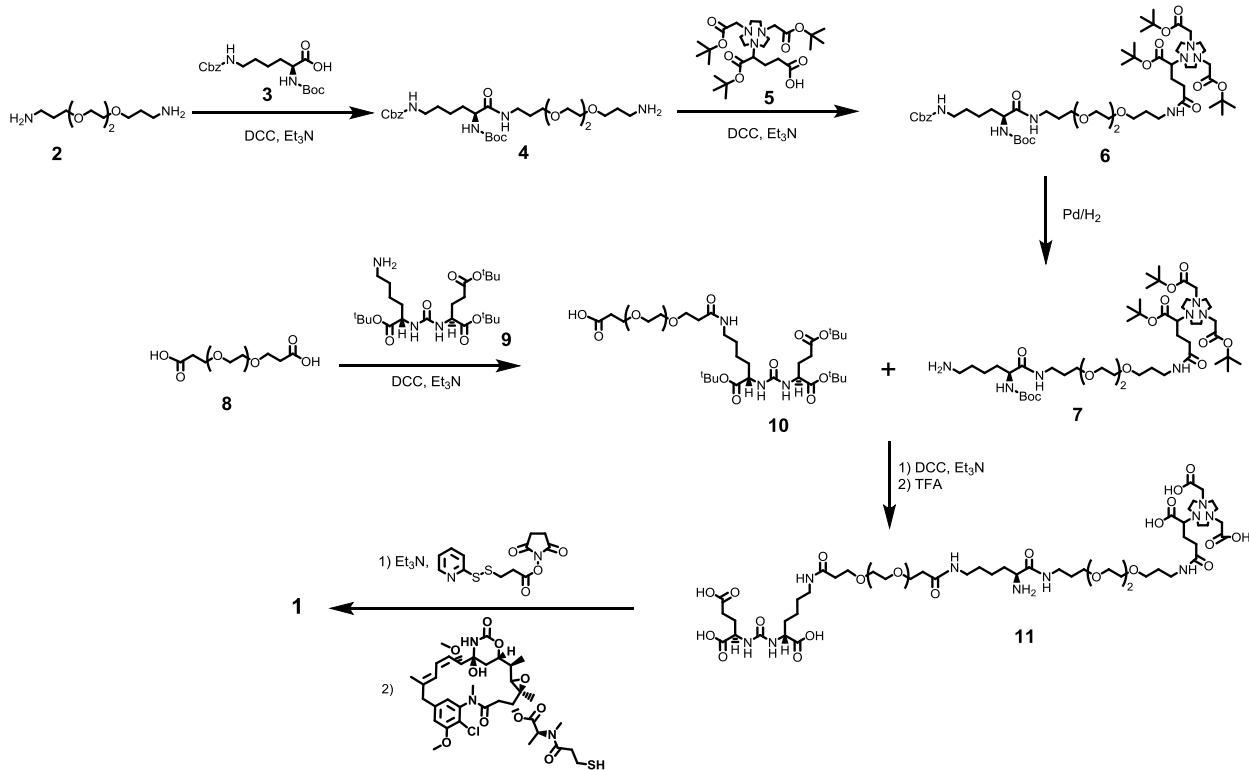
### **Work accomplished in the 3<sup>rd</sup> year toward the completion of Task 4 & Task 5**

As the continuation of last year's work, we have designed and successfully prepared a theranostic small molecule drug conjugate – T-SMDC which consists of tumor-specific ligand, PET imaging moiety, and cytotoxic drug. The resulted theranostic small-molecule drug conjugate (T-SMDC) remains the PSMA binding affinity and exhibits PSMA-dependent anti-cancer toxicity. To date, we have developed the radiochemistry procedure to label the T-SMDC with <sup>68</sup>Ga efficiently. We have started evaluating its therapeutic performance along with the concurrent PET imaging in PSMA<sup>+</sup> tumor bearing mice.

## **RESULTS**

The T-SMDC molecule design primarily consists of three parts: targeting ligand for tumor specific drug delivery, the cytotoxic drug for chemotherapy, and the radioisotope moiety for PET imaging. A linker system is also required to incorporate all three parts together with the considerations on conjugate stability & solubility, tumor targeting ability, drug potency & release, and PET imaging performance.

In this study, PSMA was selected as the biomarker for prostate cancer. Normally, the site of expression of PSMA in normal tissues is not exposed to direct blood circulation, while its expression increases by 100 – 1000 fold in prostate cancer.<sup>1</sup> PSMA expression has been positively correlated with the progression of prostate cancer stages, showing enhanced levels in metastatic and hormone-refractory prostate carcinoma.<sup>2</sup> PSMA can also form complexes with its specific ligand and result in active internalization,<sup>3</sup> which is a key factor to make the targeted therapy work by carrying and releasing the cytotoxic drugs inside prostate cancer cells. Up to date, many small organic molecules have been prepared with low-nanomolar or even subnanomolar binding affinities to PSMA.<sup>4</sup> We selected the lysine-glutamate urea PSMA specific



**Scheme 1.** NOTA-1A based scaffold for constructing SMDC

ligand reported with high tumor accumulation ( $\sim 29$  %ID/g at 1 h post injection (p.i.) in PC3-PIP tumor.<sup>5</sup> The high tumor uptake can assist to deliver desired therapeutic dose to tumors. This PSMA targeting ligand (**9**) was synthesized according to published procedure.<sup>5</sup> Tetraethylene glycol based linker (**8**) was attached to amine handle of ligand **9**, using carbodiimide chemistry to give a carboxy terminated PSMA targeting ligand **10**. The long linker separated the PSMA targeting ligand from the rest part of T-SMDC, which had been proven essential to retain its high binding affinity.<sup>4b</sup>

The effective half-life of our T-SMDC is expected to be hours (unlike days for ADCs), so the 68-min half-life of  $^{68}\text{Ga}$  (89%  $\beta^+$ ) would match its fast pharmacokinetics. The short half-life can also make daily scanning available if necessary.  $^{68}\text{Ga}$  is also a generator ( $^{68}\text{Ge}$ – $^{68}\text{Ga}$ ) based radioisotope, which will simplify the preparation and distribution by combing a radiolabeling kit design. A long-lived isotope ( $^{67}\text{Ga}$ :  $\gamma$ ,  $t_{1/2} = 3.26$  days) with identical radiochemistry can assist the *in vitro* investigation, and another surrogate PET isotope ( $^{64}\text{Cu}$ :  $t_{1/2} = 12.7$  h) can assist *in vivo* PET/CT imaging at later time points (e.g. 24 h). Also, a variety of bifunctional chelators have been developed to form stable  $^{68}\text{Ga(III)}$  complexes including three NOTA based chelators developed in our lab.<sup>6</sup>

NOTA-1A based chelator **5** was synthesized according to our previous work.<sup>6</sup> In order to link the chelator **5** to the PSMA targeting ligand, a polyethylene based di-amino linker **2** was conjugated to protected lysine **3** using carbodimide coupling to give **4** (**Scheme 1**). Free acid group of NOTA-1A **5** was reacted with **4** to yield fully protected **6**. Amine protecting group, Cbz, was removed from **6** via hydrogenation to yield scaffold for T-SMDC construction. Scaffold **7** has an amino group strategically placed for attaching with the targeting group while a protected amine group can serve as a reserved site for cytotoxic drug coupling. The presence of a free and one protected amine make scaffold **7** a versatile and flexible platform. The extended arm of ligand **10** carrying a carboxy group was coupled to amino terminated scaffold **6**. To the resultant product was isolated and deprotected using trifluoroacetic acid to give **11**. Compound **11** had six carboxy and one amino group, and this lone amino group was readily available to link the drugs.

Since we are designing a theranostic conjugate, we cannot administer a large amount of conjugates. Otherwise, the PSMA binding sites will be saturated, which will adversely affect the drug delivery efficacy and the concurrent PET imaging. Therefore, the drug embedded into the entire T-SMDC must be highly potent to make the design work. DM1, a cytotoxic maytansinoid being actively used for ADCs, has high antimitotic cytotoxicity (subnanomolar).<sup>7</sup> DM1 also meets other key criteria, such as good aqueous stability and solubility. If forming a disulfide bond via its terminal –SH group, it will be readily cleaved within the tumor environment or inside the tumor cells due to the high level of free thiols (e.g. glutathione, 1-10 mM).

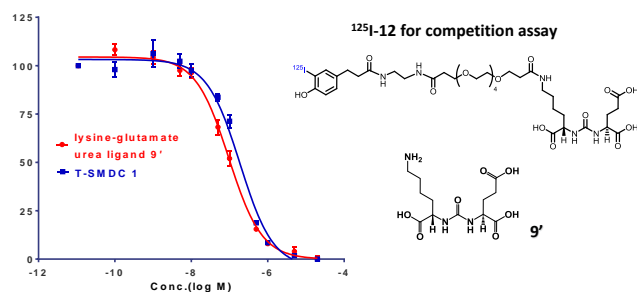
In order to link the DM1 drug to the T-SMDC, a commercially available succinimidyl 3-(2-pyridyldithio)propionate (SPDP) linker was reacted with the lone amino group in **11** to give an activated disulfide bond which was readily converted to the desired T-SMDC **1** upon reaction with the thiol terminated DM1 drug. The drug was incorporated to the entire T-SMDC at last, considering its relatively high cost and potential instability otherwise.

### Radiolabeling

NO1A-DM1 was efficiently labeled by <sup>68</sup>Ga at 60 °C in 4 M NaOAc buffer (pH 4 – 4.5 for reaction mixture) within 15 min as monitored by radio-HPLC. The radiolabeling was also tested at room temperature and 37 °C, showing incomplete reaction within the 15-min period. Another buffer of 1 M HEPES (pH 3.5) could provide similar radiolabeling efficiency, but this reaction condition would generate a much larger reaction volume. For *in vitro* and *in vivo* evaluations, the radiochemical purities of <sup>68</sup>Ga-NO1A-DM1, were maintained at over 95% as determined by radio-HPLC. Their specific activities were in the range of 50 – 80 GBq/μmol. Tested in human serum, <sup>68</sup>Ga-NO1A-DM1 remained stable (> 98%) for at least 3 h.

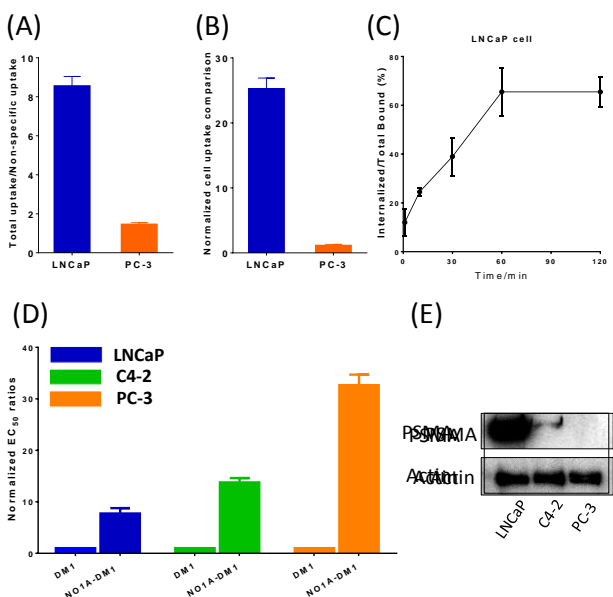
### In vitro cell assay

To retain the PSMA binding affinity, the lysine-glutamate urea PSMA specific ligand was intended to be separate away from the rest part of T-SMDC via a linker. To verify the design, the PSMA binding affinity of T-SMDC **1** was measured by competitive assay in LNCaP cells (PSMA+). The <sup>125</sup>I-labeled urea analog (<sup>125</sup>I-**12**, **Figure 1**) was used as the radioligand for the competition binding assay. Because the urea analog has no available functional group for direct radioiodination, a Bolton-Hunter moiety was introduced. The HPLC purification allowed to acquire the highest achievable specific activity for <sup>125</sup>I-**12**. The *in vitro* PSMA binding affinities of T-SMDC **1** was calculated by the measuring the concentration required to displace 50% of LNCaP cell bound <sup>125</sup>I-**12**. PSMA binding urea analog **9'** (deprotected compound **9**) served as the positive control. T-SMDC **1** inhibited the binding of <sup>125</sup>I-**12** to LNCaP cells in a dose-dependent manner as shown in **Figure 1**. T-SMDC **1** (IC<sub>50</sub>: 187 nM) presented binding affinity that was almost twice of the urea ligand **9'** (IC<sub>50</sub>: 96 nM). Although the addition of imaging and therapeutic moieties caused decrease in PSMA binding affinity, this moderate decrease was not expected to significantly compromise the *in vivo* tumor targeting for T-SMDC.



**Figure 1.** In vitro cell binding assay. The PSMA binding affinities of ligand **9** and T-SMDC **1**, were measured by a competitive cell-binding assay using PSMA positive LNCaP cell line.  $^{125}\text{I}$ -**12** was employed as the PSMA-specific radioligand. The  $\text{IC}_{50}$  values of ligand **9'** and T-SMDC **1**, were determined to be  $96 \pm 16$  and  $187 \pm 41$  nM, respectively.

The PSMA-mediated uptake and internalization of  $^{68}\text{Ga}$ -NO1A-DM1 was evaluated using LNCaP cells and PC3 cells with and without the presence of excess GPI in TBS buffer. The presence of urea ligand **9'** at the saturating concentration (1 mM) nearly abolished the cell uptake (**Figure 2A**), and the specific uptake in PSMA positive LNCaP cells was much higher than it in PSMA negative PC3 cells (**Figure 2B**), indicating that the cell uptake was mediated by PSMA. As expected,  $^{68}\text{Ga}$ -NO1A-DM1 displayed an appreciable level of internalization in a time-dependent manner in the absence of GPI (**Figure 2C**), which lays the foundation of use PSMA to internalize the drug conjugate and release the drugs within tumor cells.



**Figure 2.** In vitro cell assay. (A) Cell total uptake of  $^{68}\text{Ga}$ -NO1A-DM1 versus non-specific uptake of  $^{68}\text{Ga}$ -NO1A-DM1 with urea ligand **9'** blockage; (B) Normalized uptake of  $^{68}\text{Ga}$ -NO1A-DM1 in LNCaP and PC3 cells; the uptake in PC3 cell is set as 1.0. (C) Internalization of  $^{68}\text{Ga}$ -NO1A-DM1 in LNCaP cell; (D) Normalized  $\text{EC}_{50}$  values for DM1 and NO1A-DM1 for LNCaP, C4-2, and PC-3 prostate cancer cell lines. The  $\text{EC}_{50}$  value for DM1 for each cell line is set as 1.0, and normalized  $\text{EC}_{50}$  values for NO1A-DM1 are  $7.8 \pm 1.0$ ,  $13.8 \pm 0.9$ , and  $32.6 \pm 2.0$  for LNCaP, C4-2, and PC-3 cell lines, respectively. Cell viability was assessed by the crystal

violet staining method. The raw EC<sub>50</sub> values were acquired from three-time experiments. (E) PSMA western blot in LNCaP, C4-2, and PC-3 prostate cancer cell lines.

### Toxicity determination

The cytotoxicity of **1** was measured on three different prostate cancer cell lines with dissimilar PSMA expression levels. LNCaP and C4-2 were PSMA positive cell lines while PC3 was PSMA negative. Since the experiments were performed in different cell lines, the DM1 free drug was included into the assays as an internal standard. The acquired EC<sub>50</sub> values (half maximal effective concentration) of **1** was normalized based on the corresponding values of DM1 in each cell line (**Figure 2 A**). Due to the higher uptake of the prepared drug conjugate in PSMA positive cell lines, SMDC **1**, was confirmed to show significantly descending anti-proliferative activity ( $P < 0.01$ ) along with downward PSMA level in LNCaP > C4-2 > PC3 (**Figure 2 B**). Compared to free DM1 drug, T-SMDC **1** demonstrated reduced cytotoxicity in each cell line, which supported our hypothesis of designing drug conjugates to reduce the toxicity.

### Experiments and Methods

All chemicals, unless otherwise noted, were acquired from Sigma-Aldrich (St. Louis, MO) and used as received without further purification. All water used for buffer preparation was ultrapure (Milli-Q, Millipore, Billerica, MA) and passed through a 10 cm column of Chelex resin (Bio-Rad Laboratories, Hercules, CA) before use. NMR (Nuclear magnetic resonance) spectroscopy was performed on a Bruker 400 MHz NMR. HPLC was performed using a Waters HPLC equipped with a Waters Xterra Shield RP18 semi-prep column (250 × 10 mm, 10 μm) and read by a Waters 2996 photodiode array detector and an in-line Shell Jr. 2000 radio-detector, using a gradient of 0:100 MeCN/H<sub>2</sub>O (both with 0.1% TFA) to 100:0 MeCN/H<sub>2</sub>O within 50 min. Bulk solvents were removed using rotary evaporator under reduced pressure at 40 °C. For accurate quantification of radioactivity, experimental samples were counted for 1 min on a calibrated Perkin-Elmer (Waltham, MA) Automatic Wizard2 Gamma Counter. The <sup>68</sup>Ge/<sup>68</sup>Ga generator system was purchased from iThemba Laboratories (Somerset west, South Africa). Radiolabeled conjugates were purified by Light C-18 Sep-Pak cartridges (Waters, Milford, MA). Mass spectrum characterization was performed by LC-MS (Agilent 6540 Accurate-Mass Quadrupole Time-of-Flight LC/MS equipped with 1290 UPLC and Zorbax 300SB-C8, 5 μm column).

PC-3, C4-2, and LNCaP cells were cultured in F-12K, RPMI 1640, and DMEM respectively with 10% fetal bovine serum (FBS), 100 IU/mL Penicillin, 100 μg/mL Streptomycin, and 1% L-Glutamine in a humidified incubator with 5% CO<sub>2</sub> at 37°C. All cell culture reagents were purchased from Invitrogen.

**Compound 4** To a solution of the acid **3** (5.0 g, 13.1 mmol) in dry THF (10.0 mL) was added the solution of amine **2** (8.69 g, 39.4 mmol) dissolved in dry THF (10.0 mL). To the resulting mixture was added triethyl amine (1.59 g, 15.7 mmol) and N,N'-dicyclohexylcarbodiimide (3.25 g, 15.7 mmol). The resulting solution was stirred overnight at room temperature. The reaction mixture was then filtered and the solvent removed under vacuum to afford a crude product, which was purified by silica based flash chromatography to give colorless viscous oil **4** (1.68 g, 2.89 mmol, 22%). <sup>1</sup>H NMR (300 MHz, CHCl<sub>3</sub>): δ 1.36 (s, 9H, *t*-Bu's), 1.40-1.60 (m, 4H), 1.68 (m, 4H), 1.88 (m, 2H), 3.09 (m, 4H), 3.23 (m, 2H), 3.38-3.64 (m, 12H), 3.99 (m, 1H), 5.02 (s, 1H), 7.59 (m, 2H), 7.84 (m, 3H). <sup>13</sup>C NMR (75 MHz, CHCl<sub>3</sub>): δ 22.5, 26.3, 28.1, 28.9, 31.9, 36.7, 39.5, 40.3, 49.6, 54.7, 66.2, 68.4, 69.5, 69.9, 79.6, 127.8, 128.3, 136.6, 155.9, 156.8, 173.3. MS (ESI) *m/z* calcd for C<sub>29</sub>H<sub>50</sub>N<sub>4</sub>O<sub>8</sub>: 582.3; found: 583.3 ([M + H]<sup>+</sup>).

**Compound 6** To a solution of the acid **5** (0.5 g, 0.92 mmol) in dry THF (5.0 mL) was added the solution of amine **4** (0.64 g, 1.11 mmol) dissolved in dry THF (5.0 mL). To the resulting mixture was added triethyl amine (0.11 g, 1.11 mmol) and N,N'-dicyclohexylcarbodiimide (0.23 g, 1.11 mmol). The resulting solution was stirred overnight at room temperature. The reaction mixture

was then filtered and the solvent removed under vacuum to afford a crude product, which was purified by silica based flash chromatography to give colorless viscous oil **6** (0.62 g, 0.56 mmol, 61%). <sup>1</sup>H NMR (300 MHz, CHCl<sub>3</sub>): δ 1.12-1.56 (m, 36H, *t*-Bu's), 1.62-2.41 (m, 10H), 2.55-3.36 (m, 20H), 3.36-3.72 (m, 12H), 3.74-4.19 (m, 6H), 4.72 (m, 1H), 5.01 (s, 2H), 7.23 (m, 2H), 7.58 (m, 2H). <sup>13</sup>C NMR (75 MHz, CHCl<sub>3</sub>): δ 22.4, 25.5, 27.7, 27.8, 28.1, 28.9, 32.3, 36.7, 36.9, 40.2, 45.8, 49.5, 50.6, 54.5, 55.5, 63.3, 65.9, 66.8, 68.8, 69.7, 70.0, 79.2, 82.0, 127.5, 127.7, 128.2, 136.7, 155.7, 156.7, 171.4, 172.5. MS (ESI) *m/z* calcd for C<sub>56</sub>H<sub>97</sub>N<sub>7</sub>O<sub>15</sub>: 1107.7; found: 1108.7 ([M + H]<sup>+</sup>).

**Compound 7** Benzyloxy carbamate protected compound **6** (0.62 g, 0.56 mmol) was dissolved in 5 mL of ethanol and the solution was degassed (N<sub>2</sub>) for 5 min. To the above solution was added 10% Pd/C (10 mg). The suspension was shaken in a hydrogenator (Parr, Moline, Illinois) at room temperature for 16 h under a H<sub>2</sub> atmosphere (60 psi). The suspension was filtered through celite and the solvent was evaporated under vacuum. The obtained crude oil was purified by column chromatography to provide viscous oil **7** (0.56 g, 0.51 mmol, 91%). <sup>1</sup>H NMR (300 MHz, CHCl<sub>3</sub>): δ 1.07-1.49 (m, 36H, *t*-Bu's), 1.63 (m, 10H), 1.73-2.06 (m, 4H), 2.26 (m, 2H), 2.62-3.02 (m, 10H), 3.04-3.35 (m, 10H), 3.35-3.57 (m, 12H), 3.63-4.02 (m, 5H). <sup>13</sup>C NMR (75 MHz, CHCl<sub>3</sub>): δ 22.1, 25.4, 26.5, 27.8, 28.1, 28.8, 32.3, 37.1, 39.4, 45.8, 49.6, 50.7, 54.4, 55.6, 63.2, 66.9, 69.4, 69.9, 79.5, 82.4, 160.3, 160.6, 171.1, 172.9. MS (ESI) *m/z* calcd for C<sub>48</sub>H<sub>91</sub>N<sub>7</sub>O<sub>13</sub>: 973.6; found: 974.6 ([M + H]<sup>+</sup>).

**Compound 8** was synthesized as per the published procedure.

**Compound 9** was synthesized as per the published procedure.

**Compound 10** To a solution of the acid **8** (10.15 g, 18.5 mmol) in dry THF (10.0 mL) was added the solution of amine **9** (3 g, 6.15 mmol) dissolved in dry THF (10.0 mL). To the resulting mixture was added triethyl amine (1.24 g, 12.3 mmol) and N,N'-dicyclohexylcarbodiimide (1.77 g, 8.61 mmol). The resulting solution was stirred overnight at room temperature. The reaction mixture was then filtered and the solvent removed under vacuum to afford a crude product, which was purified by silica based flash chromatography to give colorless viscous oil **10** (1.24 g, 1.53 mmol, 25%). <sup>1</sup>H NMR (300 MHz, CHCl<sub>3</sub>): δ 1.0-1.30 (m, 27H, *t*-Bu's), 1.46-2.04 (m, 8H), 2.15-2.39 (m, 2H), 2.73-3.53 (m, 24H), 3.81-4.12 (m, 2H), 6.9-7.45 (bm, 3H, NHs), 10.16 (bs, 1H, COOH). <sup>13</sup>C NMR (75 MHz, CHCl<sub>3</sub>): δ 22.2, 27.7, 31.3, 34.7, 36.2, 38.7, 45.9, 52.7, 53.4, 66.9, 70.0, 80.2, 81.1, 81.5, 157.7, 172.1, 172.3, 173.7. MS (ESI) *m/z* calcd for C<sub>38</sub>H<sub>69</sub>N<sub>3</sub>O<sub>15</sub>: 807.5; found: 808.5 ([M + H]<sup>+</sup>).

**Compound 11** To a solution of the acid **10** (0.49 g, 0.61 mmol) in dry THF (4.0 mL) was added the solution of amine **7** (0.56 g, 0.51 mmol) dissolved in dry THF (3.0 mL). To the resulting mixture was added triethyl amine (0.06 g, 0.61 mmol) and N,N'-dicyclohexylcarbodiimide (0.13 g, 0.61 mmol). The resulting solution was stirred overnight at room temperature. The reaction mixture was then filtered and the solvent removed under vacuum to afford a crude product, which was purified by silica based flash chromatography to give colorless viscous oil with 6 *tert*-butyl groups and one Boc group. (0.31 g, 0.18 mmol, 35%). <sup>13</sup>C NMR (75 MHz, CHCl<sub>3</sub>): δ 22.2, 22.6, 24.6, 25.2, 25.6, 27.8, 28.1, 28.7, 30.2, 31.4, 31.8, 32.0, 32.9, 36.1, 37.5, 38.9, 45.8, 49.7, 50.6, 52.9, 53.4, 55.6, 56.5, 63.3, 66.9, 68.8, 69.1, 69.8, 69.9, 80.5, 81.4, 81.9, 82.5, 159.5, 159.9, 171.1, 172.2, 172.9, 173.1. MS (ESI) *m/z* calcd for C<sub>86</sub>H<sub>158</sub>N<sub>10</sub>O<sub>27</sub>: 1763.1; found: 1764.1 ([M + H]<sup>+</sup>).

To the protected conjugate (0.31 g, 0.18 mmol) was added trifluoroacetic acid (5.0 mL). The resulting solution was stirred overnight at room temperature. The solvent was then removed under vacuum to afford a crude product which was purified over reversed-phase HPLC (5% Acetonitrile/ 95% H<sub>2</sub>O to 50% Acetonitrile/ 50% H<sub>2</sub>O over 30 min, all solvent contained 0.1 % TFA), fractions containing the product were pooled and lyophilized to give colorless viscous oil

X (0.31 g, 0.15 mmol, 81%). MS (ESI)  $m/z$  calcd for  $C_{57}H_{102}N_{10}O_{25}$ : 1326.7; found: 1327.7 ( $[M + H]^+$ ).

**Compound 1** To a solution of primary amine **11** (10 mg, 7.52  $\mu$ mol) in dry DMF (1.0 mL) was added succinimidyl 3-(2-pyridyldithio)propionate (SPDP, Thermo Scientific Pierce, 3 mg, 9.61  $\mu$ mol). To the resulting mixture was added triethyl amine (1 mg, 9.9  $\mu$ mol) and the resulting solution was stirred overnight at room temperature for 6 h. The solvent was then removed under vacuum to afford a crude product which was purified over reversed-phase HPLC (5% Acetonitrile/ 95% H<sub>2</sub>O to 50% Acetonitrile/ 50% H<sub>2</sub>O over 25 min, all solvent contained 0.1 % TFA), fractions containing the product were pooled and lyophilized to give colorless viscous oil with activated disulfide bond. (6 mg, 3.84  $\mu$ mol, 51%). MS (ESI)  $m/z$  calcd for  $C_{65}H_{109}N_{11}O_{26}S_2$ : 1523.7; found: 1524.7 ( $[M + H]^+$ ).

Activated compound (5 mg, 3.3  $\mu$ mol) and the thiol terminated DM1 drug (3 mg, 4.0  $\mu$ mol) were dissolved in degassed MeOH (0.5 mL) and stirred for 6 h at room temperature. The reaction mixture was directly purified over reversed-phase HPLC (5% Acetonitrile/ 95% H<sub>2</sub>O to 50% Acetonitrile/ 50% H<sub>2</sub>O over 25 min, all solvent contained 0.1 % TFA), fractions containing the product were pooled and lyophilized to give colorless viscous oil **1** (2 mg, 1.0  $\mu$ mol, 31%). MS (ESI)  $m/z$  calcd for  $C_{89}H_{140}ClN_{13}O_{33}S_2$ : 2018.8; found: 2019.8 ( $[M + H]^+$ ).

**Compound 12** To a solution of the acid **10** (0.5 g, 0.62 mmol) in dry THF (4.0 mL) was added the solution of amine x (0.13 g, 0.62 mmol) dissolved in dry THF (4.0 mL). To the resulting mixture was added triethyl amine (0.06 g, 0.61 mmol) and N,N'-dicyclohexylcarbodiimide (0.13 g, 0.61 mmol). The resulting solution was stirred overnight at room temperature. The reaction mixture was then filtered and the solvent removed under vacuum to afford a crude product. To this, was added trifluoroacetic acid (5.0 mL). The resulting solution was stirred overnight at room temperature. The solvent was then removed under vacuum to afford a crude product which was purified over reversed-phase HPLC (5% Acetonitrile/ 95% H<sub>2</sub>O to 50% Acetonitrile/ 50% H<sub>2</sub>O over 30 min, all solvent contained 0.1 % TFA), fractions containing the product were pooled and lyophilized to give colorless viscous oil **12** (0.11 g, 0.13 mmol, 22%). MS (ESI)  $m/z$  calcd for  $C_{37}H_{59}N_5O_{16}$ : 829.4; found: 830.4 ( $[M + H]^+$ ).

**Cell Culture and Animal Model** LNCaP, PC-3, and C4-2 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The PC-3 cell line was cultured in T-media (Invitrogen Corporation, CA) supplemented with 5% fetal bovine serum (FBS) and 1  $\mu$ g Penicillin/Streptomycin (PS). LNCaP cells were cultured in RPMI 1640 media (HyClone, Thermo Scientific, IL), with 2.05 mM L-Glutamine supplemented with 10% FBS. C4-2 cell line was cultured in DMEM media (Invitrogen Corporation, CA) supplemented with 5% FBS. All cells were cultured at 37 °C in an atmosphere of 5% CO<sub>2</sub> and passaged at 75 – 90 % confluency.

**Radiochemistry** To a 1.5 mL vial containing 15  $\mu$ g of NO1A-DM1 conjugate in 90  $\mu$ L of 4.0 M NaOAc solution, 148 – 222 MBq of  $^{68}Ga^{3+}$  in 0.3 mL of 0.6 M HCl was added. The pH of the final reaction mixture was 4.0 – 4.5. The reaction mixture was incubated at 60 °C for 15 min. The radiolabeling yields were determined by radio-HPLC. The mobile phase was H<sub>2</sub>O with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B), and the gradient consisted of 20% B to 100% B in 0–20 min at 1.5 mL/min flow rate.  $^{68}Ga$ -NOTA-DM1 was eluted out at 9.8 min from the Agilent Eclipse Plus C18 column (3.5  $\mu$ m, 100  $\times$  4.6 mm).

**Serum stability assay** The in vitro stability test was performed in rat serum. Briefly,  $^{68}Ga$ -NOTA-DM1 (2 MBq, 20  $\mu$ L) was added into 400  $\mu$ L of human serum and incubated at 37 °C for 1 and 3h. A 100  $\mu$ L of sample was taken out and filtered by a 0.2  $\mu$ m filter. The resulting solution was analyzed by radio-HPLC.

**Preparation of 125I-Urea** A Pierce pre-coated iodination tube was wetted with 1 mL of Tris buffer (pH 7.5). To the pre-wetted tube was added 100  $\mu$ L of Tris buffer, followed by 5  $\mu$ L (37 MBq) of Na<sup>125</sup>I (Perkin-Elmer). The iodide was activated for 6 min at r.t. and then added to

compound **12** solution (0.5  $\mu$ g in 0.025 mL H<sub>2</sub>O). After 9 min at r.t., the mixture was directly applied to semi-preparative HPLC. HPLC fractions of 125I-... were collected between 20 and 21 min and then concentrated by a Sep-Pak® Light C18 cartridge.

**Competition assay** The PSMA binding affinities of NO1A-DM1 were determined by a competitive cell-binding assay using 125I-... as the radioligand. The urea ligand 3' was included as a positive control. Suspended LNCaP cells in Tris-buffered saline (TBS) were seeded on multi-well DV plates (Millipore) with  $5 \times 10^4$  cells per well, and then incubated with <sup>125</sup>I-12 (33,000 cpm/well) in the presence of increasing concentrations (0 – 10,000 nM) of ligand 3' and NO1A-DM1 at r.t. for 2 h (n=4). The final volume in each well was maintained at 200  $\mu$ L. At the end of incubation, unbound <sup>125</sup>I-... was removed by filtration followed by five-time rinses with cold TBS buffer. The filters were collected and their radioactivity was measured. The best-fit IC<sub>50</sub> values (inhibitory concentration where 50% of the <sup>125</sup>I-... bound on LNCaP cells were displaced) of ligand 3' and NO1A-DM1 were calculated by fitting the data with nonlinear regression using GraphPad Prism 6.0.

**Cell uptake assay** Cell uptake experiments were performed in the PMSA positive LNCaP cell line and the PMSA negative PC-3 cell line. 6-well plates were seeded with  $5.0 \times 10^5$  cells and incubated overnight in a humidified incubator at 37 °C with 5% CO<sub>2</sub>. Cells were then incubated with 500  $\mu$ L binding buffer (20mM Tris, 150mM NaCl, pH 7.4) containing  $\sim 6.0 \times 10^5$  CPM <sup>68</sup>Ga-NOTA-DM1. To account for non-specific binding additional 6-well plates were incubated with 1mM Urea ligand 9' in addition to the <sup>68</sup>Ga-NOTA-DM1. The plates were incubated at 37 °C for 1hr. After incubation, the cells were washed with cold binding buffer and then trypsinized. Trypsinized cells were placed in culture tubes and the radioactivity associated with the cells was counted in a 2480 automatic gamma counter (Perkin Elmer). The amount of cells per well was measured using a TC10 automated cell counter (Bio-Rad).

**Internalization assay** Cell internalization experiment was performed in PMSA positive LNCaP cell line. 12-well plates were seeded with  $2.0 \times 10^5$  cells and incubated overnight in a humidified incubator at 37°C with 5% CO<sub>2</sub>. Cells were washed once with binding buffer (20mM Tris, 150mM NaCl, pH 7.4) at 37°C. Cells were then incubated with  $\sim 6.0 \times 10^5$  CPM <sup>68</sup>Ga-NOTA-DM1 in 0.4mL binding buffer for 1, 10, 30, 60, and 120min. At the end of incubation cells were washed once with ice cold binding buffer and then incubated twice for 5 min with 0.5mL ice-cold low pH stripping buffer (150mM NaCl, 50mM glycine, pH 3.0) to remove surface bound <sup>68</sup>Ga-NOTA-DM1 and the fractions combined and saved in a culture tube. Cells were then incubated with 0.5mL 4M NaOH at 37°C for 15 minutes. NaOH solubilized cells that contained the internalized <sup>68</sup>Ga-NOTA-DM1 were placed in separate culture tubes. Both surface bound and internalized radioactivity were counted in a 2480 automatic gamma counter (Perkin Elmer). Internalized <sup>68</sup>Ga-NOTA-DM1 was expressed as the percent of internalized activity to the total bound activity.

**Toxicity assay (crystal violet method)** C4-2, LNCaP, and PC-3 cell lines were regularly maintained in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (FBS). For the growth curves, 4,000 cells were seeded per well in 96-well plates. 24 hours after seeding, the cells were treated with DM1 and DM1-NO1A respectively at a series of concentrations (0.3, 1, 3, 10, 30, 100, 300, 1000, and 3000 nM; n = 6 per concentration). After 36 hours of treatment, cells were washed once by PBS, then fixed in 100  $\mu$ L/well 1% glutaraldehyde (Sigma-Aldrich) for 15 minutes, then stained with 100 $\mu$ L/well 0.5% crystal violet (Sigma-Aldrich) for 15 minutes. Gently wash each well with running water. Then crystal violet was dissolved in 100  $\mu$ L/well Sorenson's solution and the relative cell number was determined by absorbance reading at 560 nm. This experiment was repeated three times in total.

**Western blot** The cell lysates were harvested, and equivalent amounts of protein were separated on Bolt 4-12% Bis-tris Plus gel and transferred to nitrocellulose membranes. The membranes

were blocked with 5% skim milk and then incubated with primary antibodies indicated overnight at 4°C. The membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies, and signals were collected by an enhanced chemiluminescent detection system (Pierce, Rockford, IL) with SuperSignal West Dura extended duration substrate kit (Thermo). The PSMA antibodies used in experiment were purchased from Abcam (cat: ab19071).

**Statistical Analysis** Statistical analyses were performed using GraphPad Prism. A p value less than 0.05 (unpaired t test) was considered statistically significant. All results are presented as mean ± standard deviation.

## KEY RESEARCH ACCOMPLISHMENTS

- We have applied the proposed bifunctional chelator scaffold system to radiolabeling of antibodies with  $^{64}\text{Cu}$  via a facile click-chemistry strategy (**published**).
- We have applied the chelator scaffold design to molecular design of targeted dual-modality imaging probes (**published**).
- A theranostic drug conjugate for prostate cancer was designed and synthesized with the integration of a PET imaging functionality (*to be published*).

## REPORTABLE OUTCOMES

1. Lo S-T, Kumar A, and Sun X: Delivery and controlled release of therapeutics via dendrimer scaffolds. Chapter 10 of “*Nanoparticle Delivery of Biotherapeutics*” edited by Vooght-Johnson. Published by Future Science Group, **2015**
2. Kumar A, Zhang S, Hao G, Hassan G, Ramezani S, Lo S-T, Sagiya K, Takahashi M, Sherry AD, Oz OK, Kovacs Z, and Sun X: Molecular Platform for Design and Synthesis of Targeted Dual-modality Imaging Probes. *Bioconjugate Chemistry*, **2015**, 26(3):549-558. *ACS Editor's Choice*
3. Kumar A, Hao G, Liu L, Ramezani S, Hsieh JT, Oz OK, and Sun X: Click-Chemistry Strategy for Labeling Antibodies with Copper-64 via a Cross-Bridged Tetraazamacrocyclic Chelator Scaffold. *Bioconjugate Chemistry*, **2015**, 26(4), 782-789.

## CONCLUSION

We have designed and synthesized the proposed bifunctional chelator scaffold system, CB-TE2A( $^t\text{Bu}$ )<sub>2</sub>-N<sub>3</sub> for the further construction of theranostic agents and multi-modality imaging probes for aggressive prostate cancer. In the meanwhile, we have extended the design concept to synthesize a bivalent imaging probe for quantitative PET imaging of PSMA expression in prostate cancer.

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